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INTERFERENCE OF SOME THIOL DERIVATIVES WITH THE PHARMACOLOGICAL EFFECTS OF ARACHIDONIC ACID AND SLOW REACTING SUBSTANCE C AND WITH THE RELEASE OF RABBIT AORTA CONTRACTING SUBSTANCES

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Arachidonic acid and Slow Reacting Substance C (SRS-C), which is an incubate of egg yolk with non-toxic concentrations of the phospholipase A-containing venom of *Naja nigricollis*, induce hypotension in rabbits and dogs, increase the pulmonary resistance to inflation in guinea pigs and release Rabbit Aorta Contracting Substance (RCS) from isolated guinea pig lungs. RCS is a new principle, initially shown to be released from isolated guinea pig lungs by bradykinin, SRS-A and anaphylactic shock. The in vivo effects, which are known to be blocked by acidic anti-inflammatory agents, were suppressed after incubation with, or administration of, sulfhydryl and antioxidant agents, whereas these antagonists suppressed the release of RCS at concentrations that did not affect the activity of RCS, once it was formed. Mechanism of action of the various antagonists and the chemical nature of RCS are discussed, in the light of results suggesting that phospholipase A plays a major role in experimental inflammation, leading to the formation of labile lipid peroxides, possibly similar to prostaglandin precursors, and which are responsible for RCS activity.

Arachidonic acid | Bronchoconstriction | Rabbit aorta contracting substances | Thiol reagents
Bradykinin | Inflammation | Slow reacting substance C

1. INTRODUCTION

I.v. administration of bradykinin, Slow Reacting Substance A (SRS-A), Slow Reacting Substance C (SRS-C) and arachidonic acid (AA), mimic some of the manifestations of acute anaphylactic shock, such as hypotension in rabbits and dogs, or increased pulmonary resistance to inflation in guinea pigs. In the rabbit, phenylbutazone and similar anti-inflammatory agents shorten hypotension induced by bradykinin (Lecomte, 1960; Vargaftig, 1966 and 1967), abolish hypotension caused by SRS-C (Vargaftig et al., 1969a) and markedly diminish hypotension caused by anaphylactic shock (Lecomte, 1966, Vargaftig et al., 1969b). The same agents also block the effects of all these agonists upon pulmonary resistance to inflation (Collier and Shorley, 1960; Berry, 1966; Berry et al., 1963; Vargaftig et al., 1969a). Moreover, anaphylactic shock, bradykinin and SRS-A release from the isolated guinea pig lung an active principle, called Rabbit Aorta Contracting Substance (RCS) that contracts the spiral strip of rabbit aorta and pulmonary artery. This release is blocked by anti-inflammatory agents (Piper and Vane, 1969). Vargaftig and Dao Hai (1971a) have shown that SRS-C and AA release a similar substance, which has been designated RCS-C. This release is equally blocked by the anti-inflammatory drugs. It has been suggested (Piper and Vane, 1969; Collier, 1969) that all agonists liable to partial or total blockade by anti-inflammatory drugs might release a common mediator; RCS has been proposed as the mediator. We have

now demonstrated that 2-mercaptoethanol and 3 other thiol derivatives block the *in vivo* effects of AA and of SRS-C and inhibit the release of RCS from guinea pig lung. New evidence is thus provided that the pharmacological activity of SRS-C and of AA is indirect and may share a common route with anaphylactic shock in the guinea pig. A possible role of RCS as a final mediator for the various agonists concerned is also reinforced.

2. MATERIALS AND METHODS

2.1. *Blood pressure and pulmonary resistance to inflation*

Dogs, rabbits and guinea pigs were prepared for recording of arterial blood pressure by means of a mercury manometer or a Statham pressure transducer connected to a Devices M2 or to a Grass No. 7 pen recorder; guinea pigs were artificially ventilated and the air overflow recorded with a Statham pressure transducer, by the method of Konzett and Rossler (1940). All animals were anaesthetized with sodium pentobarbitone, 40 mg/kg, dogs and rabbits i.v., guinea pigs i.p. Guinea pigs were pretreated with propranolol, 5–10 mg/kg, i.p., and 2.5–5 mg/kg, i.v. respectively 15–30 min and 5 min before the first injection of the agonist (Collier, 1966). When control responses of the blood pressure or of the lung resistance to the various agonists had been obtained, the antagonists were injected and followed, after 5 min, by new doses of the agonists. When blockade was apparent, the agonists were again administered every 30 min, until recovery was complete. In some experiments, a new series of agonists and antagonist was then administered. All injections were made into the jugular vein (rabbits and guinea pigs) or into the saphenous vein (dogs).

2.2. *Release of rabbit aorta contracting substances from the guinea pig lung*

Isolated guinea pig lungs from adult animals were prepared as previously described (Vargaftig and Dao Hai, 1971b), in a set-up which allowed the recording of bronchoconstriction and the responses of a cascade of isolated superfused organs to the effluent from the lungs (Vane, 1964). The lungs were placed inside a closed perspex box, where negative pressure was produced 22 times per min with a pump, allowing lung expansion and aspiration, through a tracheal cannula, of a 95% O_2 – 5% CO_2 mixture. The pulmonary artery was perfused (10 ml/min) by means of a Watson–Marlow pump with Krebs solution containing the following antagonists to prevent interference with the assays of catecholamines, histamine and acetylcholine respectively: phenoxybenzamine hydrochloride, 10^{-7} g/ml, mepyramine maleate, 10^{-6} g/ml, and atropine sulfate, 10^{-6} g/ml. Isolated superfused organs were spiral strips of aorta and pulmonary artery, to detect RCS (Piper and Vane, 1969; Vargaftig and Dao, 1971a) and strips of rat stomach, to detect prostaglandin (Ferreira and Vane, 1967). Responses of the isolated organs were checked before the experiment and throughout it, with angiotensin II 0.5–2 μg, and with prostaglandin F 2α, 0.1–1 μg, administered directly onto the cascade of isolated organs in volumes never exceeding 0.5 ml. Injections of RCS releasers and of antagonists were made into the cannulated pulmonary artery, as described under Results. Bronchoconstriction and contraction of the isolated organs were recorded on a Grass 7 Polygraph, from pressure and force transducers.

2.3. *Egg yolk coagulation test*

If egg yolk emulsions are heated to 100°C, a clot is formed in ½–1 min. Clot formation is delayed or prevented by previous incubation of these emulsions with phospholipase A (Habermann and Neumann, 1954). Crude SRS-C consisted of egg yolk emulsions (40% in volume) made up in NaCl 0.9%, mixed with 2 μg/ml of *Naja nigricollis* venom, which was used as a source of phospholipase A. The mixture was incubated for 2 h at 37°C. After incubation, the tubes were transferred to a water bath at 100°C and clot formation evaluated by the introduction of a glass rod into the emulsion at 1 min intervals, for 10 min. The increase in egg yolk coagulation time correlates with the hypotensive effects of crude SRS-C (Vargaftig and Coiron, 1971). Sulfhydryl agents, tested as antagonists, were mixed with crude SRS-C once incubation with phospholipase A had been completed, and left at room temperature for 2 min; this was followed by their i.v. injection into dogs. These incubates were also tested for clot formation at 100°C. These antagonists did not interfere with the usual clotting delay of the egg yolk emulsions, when tested

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in the concentration range used to inhibit SRS-C activity.

Partially purified SRS-C and arachidonic acid were also incubated with sulfhydryl reagents, under similar conditions, and injected into guinea pigs.

2.4. *Crude SRS-C*

Crude SRS-C consisted of egg yolk emulsions, prepared as for the egg yolk coagulation test, and injected into rabbits (0.2–0.4 ml/kg) or dogs (0.2–0.8 ml/kg). Anti-*Naja nigricollis* serum, in supramaximal concentrations (1 ml for 100 μg of venom) was added to the egg yolk incubate to stop the reaction after SRS-C had been released. Efficiency of antiserum was tested by mixing it with the venom before incubation with egg yolk had been performed; this neutralized venom completely lost its ability to release SRS-C when incubated with egg yolk. Moreover, i.v. injections of the venom, in doses equivalent to those contained in the incubate with egg yolk, did not affect the blood pressure.

2.5. *Partially purified SRS-C*

A stock solution of partially purified SRS-C (Vogt, 1957) was kept under N_2 at -15°C and dissolved with ethanol (10 mg/ml) before use. Further dilutions were made with Na_2CO_3 solution (20 mg/100 ml of distilled water).

2.6. *Drugs*

The following drugs and reagents were purchased; arachidonic acid (Nutritional Biochemicals Corporation, U.S.A.), dissolved as partially purified SRS-C; pancreatic kallikrein (Kalleone® retard, Bayer, France); bradykinin (Sandoz, Switzerland); 5-hydroxytryptamine (Sigma, U.S.A.); *Naja nigricollis* venom (Hans Hartge, Hamburg, Germany); anti-*Naja nigricollis* serum (Institut Pasteur, France); phenoxybenzamine hydrochloride (Smith, Kline and French, U.S.A.); mepyramine maleate (Rhone–Poulenc, France); atropine sulfate (Laboratoires Bruneau, France); propranolol hydrochloride (Avlocardyl ®, Laboratoires Avlon, France); phenylbutazone (Geigy, France); 2,3-dimercaptopropanol (dimercaprol, BAL, DelaLande, France); 2-mercaptoethanol, thioglycerol, 2,2′-bipyridyl, D- and D,L-penicillamine (Fluka, Switzerland); sodium diethyldithiocarbamate and dithiothreitol (Sigma, U.S.A.); 2-mercaptoethylamine hydrochloride, iodoacetic acid, L-cysteine hydrochloride, L-cystine and pyrogallol (Koch-Light, United Kingdom); sodium dimethyldithiocarbamate (Pfaltz Bauer, Switzerland); calcium ethylenediaminetetracetic acid (Laroche Navarron, France); morphine (Laboratoires Meran, France); sodium thioglycolate, N-ethylmaleimide and 5,5′-dithio-bis-(2-nitrobenzoic acid), (Calbiochem, U.S.A.); reduced glutathione (Glutathiol ®, Laboratoires Joullie, France); phentolamine (Regitine ®) and reserpine (Ciba, France); Zymofren ® (Specia, France); ascorbic acid (Hoffmann La Roche, Switzerland); glycerol (Prolabo, France) and hydroquinone (Laboratiores du Bois de Boulogne, France). Prostaglandin F2α and polyphloretin phospate were generously donated respectively by Drs A. van Dorp (Unilever, The Netherlands) and B. Hogberg (Leo Laboratories, Sweden); disodium cromoglycate (Intal ® was a gift of Fisons (United Kingdom).

3. RESULTS

3.1. *Antagonism of hypotension due to SRS-C*

3.3.1. Dogs

A list of agents tested against crude SRS-C is contained in tables 1 and 2. Threshold doses of the antagonists on the dog blood pressure were: 2-mercaptoethanol, 10 mg/kg, diethyldithiocarbamate and thioglycerol, 12.5 mg/kg, pyrogallol and hydroquinone, 0.5 mg/kg. These doses reduced the first response to SRS-C by at least 50%, but protection faded rapidly. Doses in the higher range protected for longer periods (fig. 1). Mercaptoethylamine was an effective antagonist in doses of 50–200 mg/kg, but lowered the arterial blood pressure by itself, thus rendering a quantitative evaluation difficult. Dimercaprol also induced a marked hypotension and was only used at a fixed dose of 40 mg/kg, i.p. Absorption was tested by means of its property of potentiating the hypotensive effect of bradykinin (Ferreira and Rocha e Silva, 1962), given before and 15 min after dimercaprol. A very marked potentiation of the blood pressure drop due to bradykinin, 1–4 μg/kg, was apparent. Responses to crude SRS-C under these conditions were partially or totally blocked. Partially purified SRS-C, tested on 2 dogs, markedly reduced blood pressure.

Table 1
Inhibition by sulfhydryl and anti-oxidant agents of the hypotensive effect of crude SRS-C in the dog.

Antagonist	Dose (mg/kg)*	Score of antagonistic activity***	
Mercaptoethanol	10	1.5	(6)
	35	3.6	(25)
	70	4.2	(5)
Thioglycerol	10	0.66	(3)
	25	1	(3)
	50	2.25	(4)
	100	3.33	(3)
	200	3.11	(9)
Diethyldithiocarbamate	12.5	2.5	(5)
	25	1.5	(6)
	50	3	(12)
	100	3	(12)
Dimercaprol	40**	0.76	(13)
Pyrogallol	5	1.5	(2)
	10	2.3	(3)
	20	2	(2)
	100	4	(1)

* Injection 1 min before SRS-C and at 30 min intervals.
** Injection 15 min before SRS-C.
*** Evaluated by scoring the number of injections of SRS-C that were diminished by more than 75% after the antagonist. Blockade of the first injection was scored 1; of the second, tested after 30 min, was scored 2; at 60 min 3; and similarly up to 2 hr. Total score was divided by the number of experiments per dose of antagonist, indicated between parenthesis. Score lower than 1 means that in the concerned group one or more attempts to block the hypotension more than 75% were unsucessful.

Table 2
Ineffective agents against SRS-C on rabbits and dogs.

Antagonist	Dose (mg/kg)	Pharmacological activity
Ca_2EDTA	100	Non-sulfhydryl metal quelators
Bipyridyl	50	
Phenoxybenzamine	10	Adrenergic α-blocker
Phentolamine	5	Adrenergic α-blocker
Atropine	0.5	Parasympatholytic agent
Reserpine	2	Catecholamine depletor
Zymofren®	10.000*	Kallikrein inhibitor
Polyphloretin phosphate	20	Prostaglandin inhibitor
Iodoacetic acid	25	
Disodium cromoglycate	100	Inhibitor of the release of histamine and SRS-A
Ethylmaleimide	10	
Ascorbic acid	200	
Penicillamine (D andDL)	100	Sulfhydryl reagents
L-cysteine	200	
L-cystine	400	
Thioglycolate	200	
Glutathione (reduced)	10	
DMDTC	1000	

* U/kg.

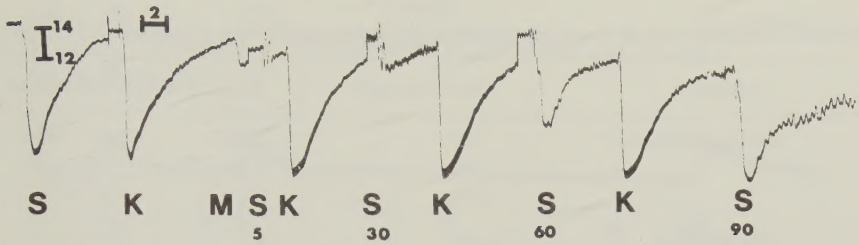

Fig. 1. Antagonism by mercaptoethanol of hypotension induced by crude SRS-C. Arterial blood pressure recorded from a 10 kg female dog. S: 0.6 ml/kg of crude SRS-C; K: 1 U of pancreatic kallikrein. At M a 35 mg/kg dose of mercaptoethanol was administered. Figures under S indicate time in minutes after the injection of the inhibitor. Observe blockade of hypotension induced by SRS-C immediately after mercaptoethanol, and gradual return after 30, 60 and 90 min. The effect of kallikrein is unaltered throughout. All injections were performed i.v. Horizontal scale: time in min; vertical scale: arterial blood pressure (cm Hg).

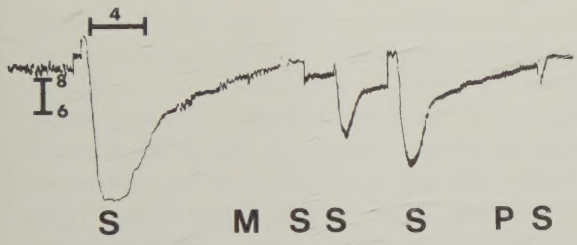

Fig. 2. Antagonism by mercaptoethanol and by phenylbutazone of hypotension induced by partially purified SRS-C. Arterial blood pressure recorded from a 6 kg female dog. S: 10 mg/kg of partially purified SRS-C; M: 35 mg/kg of mercaptoethanol; P: 5 mg/kg of phenylbutazone. Observe the blockade of hypotension induced by partially purified SRS-C immediately after mercaptoethanol, and gradual return after intervals of 30 min. Phenylbutazone also blocked the activity of partially purified SRS-C. All injections were performed i.v. Horizontal scale: time in min; vertical scale: arterial blood pressure (cm Hg).

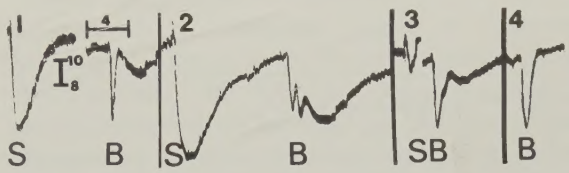

Fig. 3. Interference of penicillamine and of diethyldithiocarbamate upon the hypotensive activity of crude SRS-C and of bradykinin. Arterial blood pressure recorded from a 11 kg male dog. S: 0.5 ml/kg of crude SRS-C; B: 2 μg/kg of bradykinin. Control responses in panel 1. Panel 2: after 70 mg/kg of D-penicillamine; panel 3: after 50 mg/kg of diethyldithiocarbamate. Panel 4: after 5 mg/kg of phenylbutazone. Observe potentiation of hypotension due to bradykinin by D-penicillamine (panel 2), blockade of response to SRS-C and curtailment of response to bradykinin after diethyldithiocarbamate (panel 3), and total suppression of the secondary hypotension after bradykinin on panel 4. All injections were performed i.v. Scales as in fig. 2.

This effect was blocked by 2-mercaptoethanol and by phenylbutazone (fig. 2). Various inactive agents, including a few sulfhydryl or non-sulfhydryl metal chelators, are listed in table 2. An example (penicillamine) is provided in fig. 3, where it can also be seen that diethyldithiocarbamate, apart from inhibiting the effects of SRS-C, curtails hypotension due to bradykinin (Vargaftig and Dao Hai, 1971c).

3.1.2. Rabbits

All the agents found to be effective in the dog, suppressed the hypotension due to crude SRS-C in the rabbit (table 3). Rabbits tolerated higher doses of mercaptoethylamine and of reduced glutathione than dogs: the former completely blocked the hypotension due to SRS-C at the dose of 100 mg/kg, whereas the same dose of the latter was completely inactive, as were D,L-penicillamine, 200 mg/kg, and L-cysteine, 100 mg/kg. 2,3-Dimercaprol, 35 mg/kg, slowly administered, blocked the effects of SRS-C for 60–90 min. Calcium EDTA and 2,2-bipyridyl were ineffective when administered in doses similar to those used in dogs.

3.1.3. Antagonism of increased pulmonary resistance to inflation due to SRS-C and arachidonic acid

Table 4 summarizes the results obtained with putative inhibitors upon the effects of arachidonic acid in

Table 3
Inhibition by sulfhydryl and anti-oxidant agents of the hypotensive effect of crude SRS-C in the rabbit.

Antagonist	Dose (mg/kg)*	Score of antagonistic activity***	
Mercaptoethanol	35	0.88	(9)
	50	0.85	(7)
	200	1	(2)
Thioglycerol	100	1	(6)
	200	1.25	(15)
Diethyldithiocarbamate	50	1.4	(5)
	100	1	(6)
Dimercaprol	35	2.5	(3)
Mercaptoethylamine	100	0.7	(7)
Pyrogallol	5	0.75	(4)
	20		

* and ***: see legend to table 1.

Table 4
Interference of sulfhydryl and antioxidant agents upon the increase in pulmonary resistance to inflation due to arachidonic acid.

Drug	*n**	Dose (mg/kg)	Percentage inhibition**
Diethyldithiocar-	7	25	96
bamate	5	50	82
	12	100	81.2
Thioglycerol	6	25	51.4
	10	100	93
	14	200	95.3
Mercaptoethanol	5	25	40.3
	33	50	80.5
	28	100	87.2
Mercaptoetylamine	2	50	79
	4	100	31.5
Pyrogallol	6	0.5	75.7
	4	1	85.2
	3	5	100
Hydroquinone	3	1	46.1
	3	5	100

* Number of experiments.
** The surface of increased pulmonary resistance to inflation was evaluated by planimetry and compared before and 5 min after the i.v. administration of the antagonist.

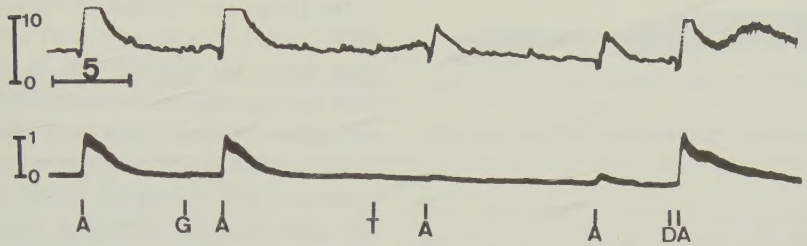

Fig. 4. Interference of thioglycerol upon the increase in pulmonary resistance to inflation and the blood pressure increase due to arachidonic acid. Arterial blood pressure (upper, cm Hg) and pulmonary resistance to inflation (lower, cm Hg) from a 450 g female guinea pig. A: 0.5 mg/kg of arachidonic acid; G, T and D: 100 mg/kg of respectively glycerol, thioglycerol and 5.5′-dithio-bis-(2-nitrobenzoic acid). Observe ineffectiveness of glycerol, suppressing activity of thioglycerol and reversion of blockade by 5,5′-dithio-bis-(nitrobenzoic acid). All injections were performed i.v.

the guinea pig bronchial resistance to inflation. The same agents which suppressed the effects of crude SRS-C in rabbits and dogs were effective in guinea pigs: mercaptoethanol, 25–100 mg/kg, diethyldithiocarbamate, 12.5–100 mg/kg, thioglycerol, 25–200 mg/kg, pyrogallol and hydroquinone, 0.5–5 mg/kg. Dimercaprol, tested on a limited number of experiments because of its toxicity, was also effective. Other agents, tested at the highest tolerable dose, were ineffective: reduced glutathione, 200 mg/kg, L-cysteine, 100 mg/kg, D- and D,L-penicillamine, 200 mg/kg, sodium thioglycolate, 100 mg/kg, and dimethyldithiocarbamate, 200 mg/kg. Finally, morphine, 5 mg/kg, ascorbic acid, 100 mg/kg, the prostaglandin antagonist polyphloretin phosphate, 100 mg/kg, and glycerol, 200 mg/kg, (fig. 4), did not interfere with the effect of arachidonic acid. Fig. 5 is an example of blockade of the effects of partially purified SRS-C by mercaptoethanol and diethyldithiocarbamate.

The moderate increase in pulmonary resistance to inflation due to prostaglandin F 2α, 100 μg/kg, was not inhibited by phenylbutazone or by the various sulfhydryl and anti-oxidant reagents.

3.1.4. Reversal of blockade

Once the blockade of hypotension due to SRS-C was established, a few thiol reagents were injected into dogs or rabbits, in an attempt to reverse blockade. L-cysteine, 100–400 mg/kg, by itself induced a marked hypertension in dogs, but did not reverse blockade by 2-mercaptoethanol, diethyldithiocarbamate or thioglycerol. The disulfide reagent 5,5′-dithio-bis-(2-nitrobenzoic acid), 50–200 mg/kg, was also ineffective, whereas iodoacetate, 10 mg/kg, injected into rabbits or dogs, reversed blockade due to thioglycerol, but since it induced hypotension by itself, the reversal was partially masked. 5,5′-Dithio-bis-(2-nitrobenzoic acid), 50–100 mg/kg, reversed the blockade induced by thioglycerol upon the effects of SRS-C or arachidonic acid in the guinea pig (fig. 4). Other antagonists were not counteracted by this reagent.

3.2. *Inhibition of the release of Rabbit Aorta Contracting Substances*

Intra-arterial injections of arachidonic acid, 10–20 μg, or SRS-C, 100–200 μg, release RCS-C from isolated guinea pig lungs (Vargaftig and Dao

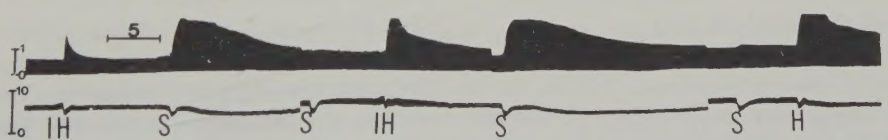

Fig. 5. Interference of mercaptoethanol and of diethyldithiocarbamate upon the increase in pulmonary resistance to inflation and the hypotension due to partially purified SRS-C. Arterial pressure (lower, cm Hg), and pulmonary resistance to inflation (upper, cm Hg) from a 480 g male guinea pig. I: 5 μg/kg of isoprenaline, immediately before H: 10 μg/kg of serotonin, to demonstrate that the animal is effectively under β-adrenergic blockade due to propranolol, injected in the beginning of the experiment (see Methods). S: 5 mg/kg of partially purified SRS-C. The 2nd S was preceded by 100 mg/kg of mercaptoethanol, and the 4th S by 100 mg/kg of diethyldithiocarbamate. Observe blockade of responses to SRS-C, with rapid return, and potentiation of responses to serotonin. All injections were performed i.v., at 15 min intervals between which the recording was stopped.

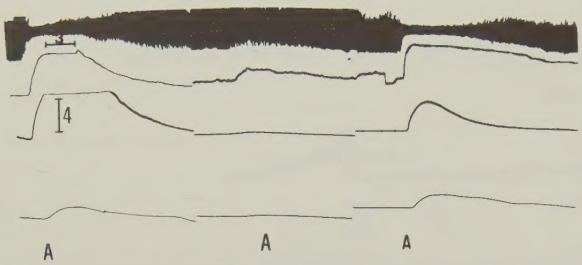

Fig. 6. Suppression by mercaptoethanol of the release of Rabbit Aorta Contracting Substance by arachidonic acid from isolated guinea pig lungs. Perfusion of the isolated lungs of a 200 g male guinea pig. Upper to lower: record of the bronchial resistance, and of the responses of superfused rabbit pulmonary artery and aorta and of the rat stomach strip. Horizontal scale: 3 min; vertical scale: 4 min. At A, 10 μg of arachidonic acid were injected to the lungs, by the pulmonary artery. The 2nd injection of arachidonic acid was preceeded by a 10 min perfusion with 40 μg/ml of mercaptoethanol, which inhibited totally the release of RCS-C. After a 30 min perfusion with mercaptoethanol-free Krebs solution, the response to arachidonic acid (3rd A) returned. The 1st and 3rd injections of arachidonic acid were performed meanwhile mercaptoethanol, at a final concentration of 40 μg/ml, was directly superfused upon the isolated organs. This excluded a direct suppressive effect of the inhibitor upon the contractions evoked by RCS-C.

Hai, 1971a). Mercaptoethanol, 40 μg/ml, 11 experiments, thioglycerol, 20–40 μg/ml, 9 experiments, and hydroquinone, 2,5–4 μg/ml, 2 experiments, perfused through the lungs for 5–10 min before arachidonic acid or SRS-C, reversibly blocked the release of RCS-C, but did not interfere with its effect upon the isolated organs (fig. 6). They were also effective when SRS-C was used in place of arachidonic acid. Sodium thioglycolate and sodium diethyldithiocarbamate (up to 80 μg/ml, 4 experiments of each), did not affect the release or the activity of RCS-C.

3.3. *Effect of incubation of antagonists upon the activity of SRS-C and of arachidonic acid*

3.3.1. Dogs

The various antagonists were added to crude SRS-C and incubated for two minutes at 37°C, after which a sample was tested for clotting and another was injected to a dog prepared for arterial blood pressure recording. Table 5 summarizes the results, showing that the various putative antagonists, when tested for in vitro activity could be divided as follows:

(a) Drugs that abolish the hypotensive activity of SRS-C, when mixed with it at concentrations lower than those that would block in vivo, and that bring back the clotting time to control values at higher concentrations. These drugs were thioglycerol, dithiothreitol and diethyldithiocarbamate, tested at concentrations of 0.2–10 mg/ml, which is equivalent to in vivo doses of 0.05–10 mg/kg, depending on the requirement of SRS-C for each individual dog. Mercaptoethanol was effective in concentrations of 10–40 mg/ml, equivalent to in vivo doses of 5–20 mg/kg. Such concentrations overlap the in vivo threshold dose of 10 mg/kg.

(b) Drugs that corrected the abnormal clotting delay without any interference with the hypotensive activity of SRS-C; for example, dimethyldithiocarbamate tested up to 100 mg/ml.

(c) Sodium thioglycolate, tested up to 100 mg/ml, did not interfere with clotting time or with the hypotensive activity.

(d) Finally hydroquinone and pyrogallol, at concentrations of 0.004–0.5 mg/ml, suppressed the hypotensive activity of SRS-C; (fig. 7) but did not affect the clotting time even at 2–10 mg/ml.

3.3.2. Guinea pigs

Incubation of dimercaprol, 1 mg/ml, and of pyrogallol, 0.2–5 mg/ml, with arachidonic acid for 2 min at room temperature resulted in a suppression of its activity upon blood pressure and pulmonary resistance to inflation in the guinea pig (fig 8).

4. DISCUSSION

Acidic anti-inflammatory, sulfhydryl and antioxidant agents share the property of inhibiting various pharmacological effects of arachidonic acid and SRS-C in 3 animal species. Since other putative inhibitors, antihistaminics, anti-inflammatory steroids or antimalarials, are ineffecive (Vargaftig et al., 1969), the antagonism is selective. The experimental anti-inflammatory activity of sulfhydryl reagents has been reported (Lecomte et al., 1953; Bailley and Sheffner, 1967), whereas clinical effectiveness of penicillamine is widely recognized (Griffin et al., 1960; Jaffe, 1965; Lorber, 1966). Penicillamine was nevertheless inactive in our experimental conditions;

Table 5
Interference of sulfhydryl and anti-oxidant agents upon the pharmacological activity of crude SRS-C.

Antagonist	Concentration (mg/ml)	Effect upon hypotensive activity*	Clotting time (min)
Thioglycerol	0.5	+	> 10
	5	+++	1
	10	++	–**
	20	+++	3
Diethyldithiocarbamate	0.05	0	–
	0.2	+	–
	0.5	0	–
	1	+++	$2\frac{1}{2}$
	2.5	+++	$1\frac{1}{2}$
	10	+++	–
Mercaptoethanol	1	+	–
	5	++	–
	20	+++	1
Dithiothreitol	25	++	4
	50	++	4
Thioglycolate	100	0	1
Dimethyldithiocarbamate	100	0	0.9
Pyrogallol	0.001	+++	> 10
	0.004	+++	–
	0.025	+++	–
	0.1	++	–
	0.25	++	> 10
	0.005	0	> 10
	0.02	0	–
	0.1	++	–
	0.5	+++	–
	2	+++	> 10
Hydroquinone	0.005	+	> 10
	0.02	+++	–
	0.1	+++	> 10

SRS-C consisted of a mixture of egg yolk suspension with 2 μg/ml of *Naja nigricollis* venom incubated for 1 hr at 37°C, to which was added 0.5 ml of serum anti-*Naja* to stop the reaction. Clotting time at 100°C for this incubate was more than 10 min. The various antagonists were mixed with SRS-C for 2 min at 37°C and then injected into a dog, whose arterial blood pressure was recorded. At the same time samples were transfered to assay tubes, and placed in a water bath at 100°C. Clotting time was measured as described in Methods. Concentrations of putative antagonists were at least 10 times lower than final doses which were effective in vivo, except for 2-mercaptoethanol, where overlaping of in vivo and in vitro effective doses and concentrations, respectively, was apparent.

* Inhibition of hypotensive activity was scored as follows: + = 25–50% inhibition; ++ = 50–75% inhibition; +++ = 75–100% inhibition.

** not performed.

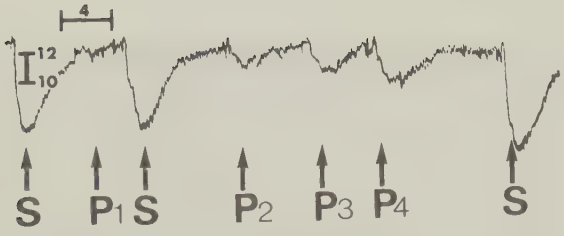

Fig. 7. Suppression by pyrogallol of the hypotensive activity of crude SRS-C. Arterial blood pressure from a 15 kg male dog. S corresponds to injections of 0.5 mg/kg of crude SRS-C. P1 – P4 indicate injections of incubate of SRS-C with pyrogallol (2 min at 37°C). Concentrations of pyrogallol, from P1 to P4 were 0.5, 0.1, 0.02, 0.005, respectively. Note the graded effect of inhibition of SRS-C activity. Scale as in fig. 2.

this seems to discard a direct relationship between clinical effectiveness in man and blockade of the effects of SRS-C or of arachidonic acid in animals. However it must be noted that anti-inflammatory effect of penicillamine is usually tested after chronic treatment, and thus may not be due to a direct action of this compound.

The demonstration that arachidonic acid and SRS-C release Rabbit Aorta Contracting Substances from guinea pig lungs and that this effect is blocked by anti-inflammatory drugs, raised the possibility that RCS consisted of precursors or derivatives of fatty acids, initially released by phospholipase A activation (Vargaftig and Dao Hai, 1971a). The recent demonstration (Vane, 1971; Ferreira et al., 1971; Smith and Willis, 1971), that aspirin and its analogues block the synthesis of prostaglandins E2 and F 2α from arachidonic acid, together with the fact that PGE2 was found in inflamed tissues (Willis, 1970), strengthen the hypothesis that somewhere in the chain of events that follows induction of experimental inflammation, prostaglandins are formed. The fact that anti-inflammatory drugs prevent their synthesis is nevertheless only circumstantial evidence that those lipid derivatives play a role in provoking inflammation.

We have recently shown (Vargaftig and Dao, 1971c) that the secondary hypotension evoked by bradykinin in rabbits is suppressed by sulfhydryl and antioxidant reagents, despite the recognized potentiation of the effects of bradykinin in these agents (Ferreira and Rocha e Silva, 1962; Erdös and Wöhler, 1963). This paradoxical situation is dependent upon the actual dose of the sulfhydryl reagents; an example is provided in fig. 3. These inhibitors also block bronchoconstriction and release of RCS by bradykinin (Vargaftig and Dao Hai, 1971c). That three series of pharmacological agents, acidic anti-inflammatory, sulfhydryl and antioxidants, interfere similarly with the effects of agonists as unrelated chemically as arachidonic acid and SRS-C on one side, and bradykinin on the other, reinforced the hypothesis that a common mechanism is involved in the pharmacological effects of these agonists. Release of RCS is an obvious canditate, since it was blocked by all the antagonists which were effective in vivo with the only exception of diethyldithiocarbamate. However, diethyldithiocarbamate does not contain a free -SH group and may only behave as an antagonist after biotransformation, which could not occur in vitro. Arachidonic acid is the precursor of prostaglandins E_2 and F 2α (Anggard and Samuelsson, 1965) and SRS-C is a mixture of unsaturated fatty acids split by venom phospolipase A from the β-position of egg lecithin (Vogt, 1957; Vargaftig and Coiron, 1971). Ineffectiveness of poly-

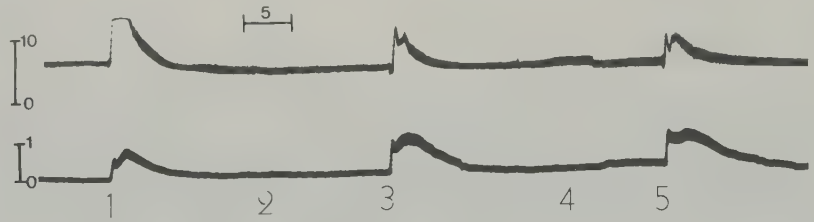

Fig. 8. Suppression by pyrogallol of the effect of arachidonic acid upon the blood pressure and the pulmonary resistance to inflation in the guinea pig. Arterial pressure (upper, cm Hg) and pulmonary resistance to inflation (lower, cm Hg) from a 420 g female guinea pig. 1 and 3: responses to 0.5 mg/kg of arachidonic acid; 2: response to an incubate of arachidonic acid with 1 mg/ml of pyrogallol; 4: response to an incubate of arachidonic acid with 0.2 mg/ml of pyrogallol; 5: response to an incubate of arachidonic acid with 0.02 mg/ml of pyrogallol. Incubation performed as described in Methods.

phloretin phosphate to interfere with the actions of SRS-C and of arachidonic acid, demonstrates that the active principle is not one of those prostaglandins but does not reject the possibility of other derivatives being involved. RCS-C is not PGF 2α or PGE 2, nor apparently one of their catabolites, because

(a) PGE 2 does not evoke bronchoconstriction;

(b) the feeble bronchoconstriction induced by PGF 2α is not inhibited by the antagonists tested;

(c) the effects of those prostaglandins do not match with those of RCS upon isolated vascular and gastrointestinal organs.

Biosynthesis of prostaglandins is a stepwise process, and cyclic and unstable peroxides are recognized intermediates (Anggard and Samuelsson, 1965). Lipid peroxides are biologically active materials, markedly toxic for animals, as is SRS-C (Delezenne and Ledebt, 1911; Boquet et al., 1950). Dakhil and Vogt (1962) and Jaques (1965) have demonstrated that arachidonic acid only contracts isolated guinea pig ileum after peroxidation, and that fresh solutions are inactive. In our hands no difference was found between fresh arachidonic acid solutions and those kept for some days in the refrigerator, but whatever the conditions of the acid before being administered to the animals, in vivo peroxidation is a mandatory step for prostaglandin formation. Rabbit Aorta Contracting Substance is relatively unstable, with a half-life of less than 5 min (Piper and Vane, 1969), this is compatible with it being one of those lipid peroxides which are active by themselves before being transformed to prostaglandins. In fact, Gryglewski and Vane (1971) have recently suggested that RCS released from AA is a prostaglandin precursor, possibly the cyclic peroxide. Blockade by anti-inflammatory, sulfhydryl and antioxidant derivatives of the release of RCS would correspond to the effect of aspirin and indomethacin in blocking the in vitro synthesis of prostaglandins from arachidonic acid (Vane, 1971).

Interference of mono- and dithiols with prostaglandin synthesis from arachidonic acid has been reported to produce a shift from PGE2 to PGF2α or *vice versa*, depending upon the -SH agent and on the presence of Cu^{2+}. The site of action appears to be on the last reaction that leads from the cyclic peroxide to PGE2 or to PGF2α; a shift from the latter to the former is brought by increased concentrations of reduced glutathione (Lands et al., 1970). Glutathione was also present in the experiments of Gryglewski and Vane (1971), where RCS was tentatively identified to the above-mentioned cyclic peroxide. It is noteworthy that reduced glutathione was inactive in our in vivo experiments, where other -SH containing agents markedly counteracted the effects of SRS-C and of AA.

Whether this was due to the limited amounts of glutathione that could be administered, without marked hypotensive effects or to the rapid clearance from the circulation or to other factors – as a dissociation between release of RCS from isolated lungs and in vivo effects of SRS-C and AA, despite all other similarities regarding inhibitors – cannot be firmly stated at this stage. Nevertheless, the finding that pyrogallol and other agents inactivated AA and SRS-C, indicates that probably an oxidation product is the actual inducer of the biological effects, but does not reject the possibility that the fate of SRS-C and of AA after in vivo administration is also oxidative, this step being precisely blocked by sulfhydryl and anti-inflammatory agents. It is noteworthy that drugs such as cysteine or penicillamine, despite reversing the prolonged clotting time of SRS-C, did not interfere with its pharmacological activities, whereas pyrogallol and hydroquinone, very active in blocking the pharmacological activities, did not reverse the effect on clotting times. This dissociation indicates that the prolonged clotting time is not dependent upon the presence of free unsaturated fatty acids or derivatives in the incubate. Once SRS-C has been released, correction of the clotting time is possible without suspension of SRS-C activity. The sole correlation found was between prolongation of clotting time and release of SRS-C.

Other possible mechanisms of action of thiols against SRS-C and AA are: (a) metal chelation, which can be discarded by the ineffectiveness of recognized chelators to antagonize SRS-C or arachidonic acid; (b) competition with -SH groups of enzymes involved with the release of the hypotensive factors, which is certainly discarded by the ineffectiveness of various sulfhydryl reagents, as penicillamine, cysteine, glutathione thioglycolate; (c) reduction of vital disulfide bridges of enzymes involved in the bio-transformation of the lipidic agonists or in the release of the mediators, which may be discarded for the same reasons mentioned in (b).

We have found (Vargaftig, unpublished) that thiol

agents are effective in blocking rat paw oedema due to carrageenin injection, and in delaying ultraviolet light-induced erythema in the guinea pig. Carrageenin-induced inflammation in the rat is followed by the appearance of prostaglandins (Willis, 1970) and it has been recently suggested by Anderson et al. (1971) that phospholipases released by leucocytes attack cell membrane phospholipid to give arachidonic acid, which is converted to prostaglandin by freely available tissue enzymes. Phospholipase A, present in the membrane of various cells (Uvnas, 1958) splits unsaturated fatty acids from the β-position of phospholipids and is the enzyme which releases SRS-C from egg yolk incubates. If phospholipase A displays an important role in inflammation (Anderson et al., 1971; Vargaftig and Dao, 1971a,b), by supplying unsaturated fatty acids that are transformed into prostaglandins, through reactions where active peroxides are formed, the hypothesis that acidic and other anti-inflammatory agents act by antagonizing the release of those peroxides, among which RCS, appears sound. Acceptance awaits nevertheless direct evidence.

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REFERENCES

Anderson, A.J., W.E. Brocklehurst and A.L. Willis, 1971, Evidence for the role of lysosomes in the formation of prostaglandins during carrageenin-induced inflammation in the rat, Pharmacol. Res. Commun. 3, 13.

Anggard, E. and B. Samuelsson, 1965, Biosynthesis of prostaglandins from arachidonic acid in guinea pig lung, J. Biol. Chem. 210, 3518.

Bailley, K.R. and A.L. Sheffner, 1967, The reduction of experimentally induced inflammation by sulfhydryl compounds, Biochem. Pharmacol. 16, 1175.

Berry, P.A., H.O.J. Collier and J.A. Holgate, 1963, Bronchoconstriction action in vivo of SRS-A and its antagonism, J. Physiol., London 165, 41P.

Berry, P.A., Slow Reacting Substance in Anaphylaxis (SRS-A) its release, actions and antagonism, Ph.D. Thesis, London, 1966.

Boquet, P., M. Dworetzky and H.E. Essex, 1950, Physiologic responses of certain animals and isolated preparations to mixtures of snake venom and egg yolk, Am. J. Physiol. 161, 561.

Collier, H.O.J. and F.G. Shorley, 1960, Analgesic antipyretic drugs as antagonists of bradykinin, Brit. J. Pharmacol. Chemotherap. 15, 601.

Collier, H.O.J., 1966, in: Hypotensive Peptides, eds. E.G. Erdos, N. Back, and F.I. Sicuteri (Springer Verlag, N.Y.) p.305.

Collier, H.O.J., 1969, New light on how aspirin works, Nature, London 223, 35.

Dakhil, T. and W. Vogt, 1962, Hydroperoxyde als Trager der darmerregenden Wirkung hochungesattigter Fettsauren, Arch. Exptl. Pathol. Pharmakol. 243, 174.

Delezenne, M.C. and S. Ledebt, 1911, Formation de substances hemolytiques et de substances toxiques aux depens du vitellus de l'oeuf soumis a l'action du cobra, Compt. Rend. 1, 81.

Erdős, E.G. and J.R. Wöhler, 1963, Inhibition in vivo of the enzymatic inactivation of bradykinin and kallidin, Biochem. Pharmacol. 12, 1193.

Ferreira, S.H. and M. Rocha e Silva, 1962, Potentiation of bradykinin by dimercaptopropanol (BAL) and other inhibitors of its destroying enzymes in plasma, Biochem. Pharmacol. 11, 1123.

Ferreira, S.H. and J.R. Vane, 1967, Prostaglandins: their disappearance from and release into the circulation, Nature, London 216, 868.

Ferreira, S.H., S. Moncada and J.R. Vane, 1971, Indomethacin and aspirin abolish prostaglandin release from the spleen, Nature, London 231, 237.

Griffin, S.W., A. Ulloa, M. Henry, M.L. Johnston and H.L. Holley, 1960, In vivo effect of penicillamine on circulating rheumatoid factor, Clin. Res. 8, 87.

Gryglewski, R. and J.R. Vane, 1971, Rabbit Aorta contracting substance (RCS) may be a prostaglandin precursor, Brit. J. Pharmac. Chemotherap. 43, 420P.

Habermann, E., and W. Neumann, 1954, Die Hemmung der Hitzekoagulation von Eigelb durch Bienengift-ein Phospholipase Effekt, Hoppe Seyl. Z., 297, 179.

Jaffe, I.A., 1965, The effect of penicillamine on the Laboratory Parameters in Rheumatoid Arthritis, Arthritis Rheumatism 8, 1064.

Jaques, R., 1965, Suppression by morphine and other analgesic compounds of the smooth muscle contraction produced by arachidonic acid peroxyde, Helvet. Physiol. Acta 23, 156.

Konzett, H. and R. Rossler, 1940, Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur, Arch. Exptl. Pathol. Pharmakol. 195, 71.

Lands, W., R. Lee and W. Smith, 1970, in: Prostaglandins, eds. W. Ramwell and J.E. Shaw (Ann. N.Y. Acad. Sci.) p.107.

Lecomte, J., H. van Cauwenberge, J. Goblet, and M. Uliers, 1953, Action inhibitrice comparee de la cysteinamine et de ses derives sur l'oedeme au blanc d'oeuf chez le rat, Annls. Endocr. 14, 123.

Lecomte, J., 1960, Antagonisme entre bradykinine synthetique et phenylbutazone chez le lapin, C. R. Soc. Biol., Paris 154, 2389.

Lecomte, J., 1966, Sur l'activite anti-anaphylactique des derives de la Pyrazolone chez le lapin, Arch. Intern. Pharmacodyn. 161, 463.

Lorber, A., 1966, Penicillamine therapy for rheumatic lung disease: effects on protein sulfhdryl groups, Nature, London 210, 1235.

Piper, P.J. and J.R. Vane, 1969, Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs, Nature 223, 29.

Smith, J.B. and A.L. Willis, 1971, Aspirin selectively inhibits prostaglandin production in human platelets, Nature, London 231, 235.

Uvnas, B., 1958, The mechanism of histamine liberation, J. Pharm. Pharmacol. 10, 1.

Vane, J.R., 1964, The use of isolated organs for detecting active substances in the circulating blood, Brit. J. Pharmacol. Chemotherap. 23, 360.

Vane, J.R., 1971, Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs, Nature, London 231, 235.

Vargaftig, B.B., 1966, Effets des analgesiques non narcotiques sur l'hypotension due a la bradykinine, Experientia, Basel 22, 182.

Vargaftig, B.B., 1967, Antagonisme par les analgesiques non narcotiques de la liberation de kinines plasmatiques due au venin de *B. jararaca* et la kallikreine pancreatique, Med. Pharmacol. Exptl. 17, 517.

Vargaftig, B.B., E.P. Miranda and B. Lacoume, 1969a, Inhibition by non-steroidal anti-inflammatory agents of in vivo effect of Slow Reacting Substance C, Nature, London 222, 883.

Vargaftig, B.B., N. Bhargava, C.J. de Vos and I.L. Bonta, 1969b, Interference of carrageenin, non-steroidal anti-inflammatory drugs and protease inhibitors with the kallikrein kinin system, Pharmacol. Res. Commun. 1, 203.

Vargaftig, B.B. and M. Coiron, 1971, Detection de la phospholipase A de divers venins animaux par ces effets sur la coagulation du jaune d'oeuf et la formation de Substance a Contraction Differee C, J. Pharmacol., Paris 2, 155.

Vargaftig, B.B. and N. Dao Hai, 1971a, Release of vaso active substances from guinea pig lungs by Slow Reacting Substance C and arachidonic acid: its blockade by nonsteroid antiinflammatory agents, Pharmacology 6, 99.

Vargaftig, B.B. and N. Dao Hai, 1971b, Pharmacologie, mode d'action et antagonisme de la 'Substance à Contraction Différée C' libérée par la phospholipase A à partir du jaune d'oeuf, J. Pharmacol., Paris 3, 287.

Vargaftig, B.B., and N. Dao Hai, 1971c, Paradoxical inhibition of the effects of bradykinin by some sulfhydryl reagents, Experientia, in press.

Vogt, W., 1957, Pharmacologically active substances formed in egg yolk by cobra venom, J. Physiol., London 136, 131.

Willis, A.L., 1970, Identification of prostaglandin E2 in rat inflammatory exudate, Pharmacol. Res. Commun. 2, 297.